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Antimutagenic; differentiation-inducing; and antioxidative effects of fragrant ingredients in Katsura-uri (Japanese pickling melon; *Cucumis melo* var. *conomon*)

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ABSTRACT

Six fragrant ingredients were identified in fully-ripened Katsura-uri (Japanese pickling melon; *Cucumis melo* var. *conomon*). Four of them were sulfur-containing compounds [methylthioacetic acid ethyl ester (MTAE), acetic acid 2-methylthio ethyl ester (AMTE), 3-methylthiopropionic acid ethyl ester (MTPE), and acetic acid 3-methylthio propyl ester (AMTP)]; and the others were benzyl acetate and eugenol. The newly identified MTAE and AMTP possessed antimutagenic activity as determined by their ability to inhibit the UV-induced mutation in repair-proficient *E. coli* B/r WP2. MTAE and MTPE (esters with thiocarbonic acid and alkyl alcohol) induced the differentiation of human colon cancer cells (RCM-1 cells), but AMTE and AMTP (esters with carbonic acid and thioalkyl alcohol) did not. A specific thioester motif containing a thiocarbonic acid and alkyl alcohol correlated with these compounds ability to induce differentiation. AMTE, MTPE, AMTP, and eugenol had higher oxygen radical absorbing capacity than the antioxidative vitamin, ascorbic acid. The quantity of MTPE, AMTP and eugenol increased 49-fold, >1175-fold and 11-fold, respectively, in the fully-ripened fruit as compared to the mid-ripened fruit.

1. Introduction

Katsura-uri, an heirloom vegetable in Kyoto (Japanese pickling melon; *Cucumis melo* var. *conomon*), is an unsweet fruit, however, possesses an intense muskmelon-like fragrance in the ripening process. Although the principal fragrant ingredients in some variety of melons are known to be esters and thioesters [1–4], the antimutagenic activities of these compounds are not known. One strategy for efficacious chemoprevention and chemotherapy would be to find chemicals that reduce the risk of cancer through various mechanisms, such as preventing mutational events, induction of differentiation in transformed cells, and inhibition of oxidative stress. The primary objective of this study is to identify antimutagenic and anticarcinogenic compounds from the fragrant ingredients of Katsura-uri as determined by their ability to inhibit UV-induced mutation in *E. coli* B/r WP2, to induce differentiation in RCM-1 human colon cancer cells, and to show antioxidative activity. In our previous study, an *n*-hexane extract of fully-ripened Katsura-uri exhibited antimutagenic activity, which was assessed by UV-induced mutation assays using *E. coli* B/r WP2 [5]. One active compound, 3-methylthiopropionic acid ethyl ester (MTPE), was identified as an anticarcinogenic compound as determined by its ability to induce differentiation in RCM-1 cancer cells [6]. MTPE was isolated from the fragrant fraction of Katsura-uri by *n*-hexane extraction and silica-gel chromatography [6]. In this paper, a global chemical structure identification analysis was used to identify potential new anticarcinogenic compounds from the fragrant fraction in Katsura-uri fruit. The global chemical structure identification analysis used gas chromatography–mass spectrometry to identify unique compounds in the fragrant fraction, and then these compounds were assessed for anticarcinogenic properties as a function of the following biological endpoints: antimutagenic activity, inductive potential of differentiation and antioxidative activity. We then determined at which ripening stages of the fruit (fully-ripened and mid-ripened), produced the highest levels of the anticarcinogenic compounds.

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2. Materials and methods

2.1. Materials

3-Methylthiopropionic acid ethyl ester (MTPE; 98% pure grade, CAS #13327-56-5), methylthioacetic acid ethyl ester (MTAE; 98% pure grade, CAS #4455-13-4) were purchased from Avocado Research Chemicals Ltd. (Lancashire, England). 2-Methylthio ethanol (CAS #5271-38-5), 3-methylthio-1-propanol (CAS #505-10-2), benzyl acetate (BA; CAS #140-11-4), eugenol (CAS #97-53-0), and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH; CAS #2997-92-4) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; CAS #258-422-8) was purchased from Calbiochem (San Diego, CA, USA). Fluorescein sodium salt (CAS #518-47-8) was purchased from Sigma-Aldrich, Inc (St Louis, MO, USA). Katsura-uri (Japanese pickling melon, *Cucumis melo* var. *conomon*) was harvested after it was ripened in August, 2009 in an open field culture system at the Kyoto Prefectural Agricultural Research Institute (Kameoka, Japan). Mid-ripened Katsura-uri was identified when the fruit reached 25 cm in length. Fully-ripened Katsutra-uri was identified when the fruit color changed from pale-green to white, and fragrance can be detected by a human sense of smell at the bottom of the fruit.

2.2. Identification of fragrant ingredients in Katsura-uri

Katsura-uri fruit (3.5 kg) was homogenized and extracted twice with methanol (1.5 L) at room temperature for 1 h. The filtered methanol extract was partitioned with *n*-hexane. The *n*-hexane layer was evaporated to dryness under 40 °C. The *n*-hexane extract (180 mg), was further chromatographically fractioned on a silica-gel column (Merck; Silica gel 60, 35–70 mesh, 5 g; Ø 0.8 × 24 cm). The sample was eluted from the silica-gel with 50 mL of each eluent: *n*-hexane, and 2.5% acetone in *n*-hexane. The fraction that was eluted by 2.5% acetone in *n*-hexane (56 mg) had a very specific melon-like fragrance as determined by a human sense of smell (named the fragrant fraction of Katsura-uri). This fragrant fraction was analyzed with gas chromatography-electron ionization-mass spectrometry (GC–EI-MS; JEOL JMS-AMSUN200 mass spectrometer, coupled on a Hewlett-Packard 6890 gas chromatograph). The capillary column was a DB-5 (25 m × 0.2 mm, 0.33 μm film thickness; J&W Scientific, Folsom, CA, USA). The column oven temperature was held at 60 °C for 5 min and then was increased to 250 °C at a rate of 5 °C/min.

2.3. Synthesis of acetic acid 2-methylthio ethyl ester (AMTE) and acetic acid 3-methylthio propyl ester (AMTP)

Acetic acid (6.0 g) and sulfuric acid (1.5 ml) were mixed with 4.0 g of 2-methylthio ethanol or 3-methylthio-1-propanol for syntheses of acetic acid 2-methylthio ethyl ester (AMTE) or acetic acid 3-methylthio propyl ester (AMTP), respectively. Each mixture was placed in an Erlenmeyer flask and kept at 70 °C on a water bath for 1 h. The solution was then cooled and neutralized by sodium hydrogen carbonate. The product was washed with distilled water (50 ml, three times), and then extracted with *n*-hexane. The yield of AMTE and AMTP products were about 1.5 g. The purity of the synthesized AMTE and AMTP was >98%, which was confirmed by GC–EI-MS, and HPLC analyses. ^{1}H NMR, and ^{13}C NMR spectra were obtained on a JEOL GX-270W using $CDCL_3$ as the solvent with TMS as the internal standard. These NMR-data of AMTE and AMTP are presented below:

AMTE. Clear liquid (25 °C). EI-MS *m/z* (relative intensity): 134 $[M]^+$ (1), 91 $[M\text{-}CH_3CO]^+$ (4), 87 $[M\text{-}CH_3S]^+$ (5), 74 $[M\text{-}CH_3SCH]^+$ (5), 61 $[M\text{-}CH_3COOCH_2]^+$ (100), 47 $[M\text{-}CH_3COOCH_2CH_2]^+$ (35). ^{1}H NMR ($CDCL_3$, 270 MHz): δ 2.08 (3H, s, H-1), 2.16 (3H, s, H-5), 2.72 (2H, t, $J_{4,3}$ = 15.6, H-4), 4.24 (2H, t, $J_{3,4}$ = 15.6, H-3). ^{13}C NMR ($CDCL_3$, 67.8 MHz): δ 15.88 (C-5), 20.99 (C-1), 32.62 (C-4), 63.03 (C-3), 170.67 (C-2).

AMTP. Clear liquid (25 °C). EI-MS *m/z* (relative intensity): 148 $[M]^+$ (14), 88 $[M\text{-}CH_3SCH]^+$ (100), 61 $[M\text{-}CH_3COOCH_2CH_2]^+$ (47), 47 $[M\text{-}CH_3COOCH_2CH_2CH_2]^+$ (15). ^{1}H NMR ($CDCL_3$, 270 MHz): δ 1.93 (2H, tt, $J_{3,2}$ = 6.35, $J_{3,4}$ = 7.25, H-3), 2.06 (3H, s, H-1), 2.11 (3H, s, H-5), 2.56 (2H, t, $J_{4,3}$ = 7.25, H-4), 4.16 (2H, t, $J_{2,3}$ = 6.35, H-2). ^{13}C NMR ($CDCL_3$, 67.8 MHz): δ 15.58 (C-6), 21.02 (C-1), 28.23 (C-4), 30.65 (C-5), 63.05 (C-3), 170.86 (C-2).

2.4. Quantification of fragrant ingredients in Katsura-uri

Katsura-uri was stored at 4 °C until all analyses were performed on the day after harvest. All Katsura-uri were washed with water and cut into four equal pieces longitudinally, and therefore each piece represents the whole Katsura-uri for quantification of fragrant ingredients. One of the pieces of Katsura-uri was put into an automatic homogenizer (CQM-V2, Toshiba). The homogenates (300 g) were each extracted three times with 70 mL of *n*-hexane. The *n*-hexane extracts were combined and absorbed to the silica-gel cartridge column (Waters, Sep-pak, catalogue number WAT020520), and washed with 3.0 mL of *n*-hexane, and then a fragrant fraction was eluted with 3.0 mL of 2.5% acetone in *n*-hexane and the fragrant fractions were collected for the identification of the organic compounds. The GC–EI-MS in the selected ion monitoring (SIM) mode using selective fragment ions was used to quantify each fragrant ingredient. EI-MS *m/z* (relative intensity) of MTAE, AMTE, MTPE, AMTP, BA and eugenol are presented in the following: MTAE 134 $[M]^+$ (37), 88 (35), 61 (100), 45 (19); AMTE 134 $[M]^+$ (1), 91 (4), 87 (5), 74 (5), 61 (100), 47 (35); MTPE 148 $[M]^+$ (63), 133 (2), 119 (8), 103 (39), 87 (15), 74 (5), 61 (100), 47 (30); AMTP 148 $[M]^+$ (14), 88 (100), 61 (47), 47 (15); BA 150 $[M]^+$ (22), 108 (100), 91 (74), 90 (48), 79 (14), 77 (9), 65 (17), 51 (14); eugenol 164 $[M]^+$ (100), 149 (41), 131 (41), 121 (32), 103 (58), 91 (53), 77 (15), 65 (21), 55 (30). As the peaks of MTAE and AMTE in GC–MS partially overlapped with each other at t_R 12.8–13.2 min, selected ions for monitoring of MTAE and AMTE should be specifically different. The relative intensity of ion peak 88 of MTAE and AMTE were 35% and <0.5%, respectively, and the SIM for MTAE quantification in GC–MS was decided to be 88. The relative intensity of ion peak 74 of AMTE and MTAE were 5% and <0.01%, respectively, and the SIM for AMTE quantification in GC–MS was decided to be 74. As the peaks of MTPE and AMTP in GC–MS partially overlapped with each other at t_R 17.1–17.6 min, selected ions for monitoring of MTPE and AMTP should also be specifically different. The relative intensity of ion peak 103 of MTPE and AMTP were 39% and <0.5%, respectively, and the SIM for MTPE quantification in GC–MS was decided to be 103. The relative intensity of ion peak 88 of AMTP and MTPE were 100% and <2%, respectively, and the SIM for AMTP quantification in GC–MS was decided to be 88. Because BA and eugenol and each parent ion had different retention times, 150 for BA and 164 for eugenol were selected. For the reasons considering above, in the SIM mode, integration of selected ion intensity (*m/z* 88 for 11.3–13.3 min in MTAE, 74 for 12.4–14.4 min in AMTE, 103 for 16.0–18.0 min in MTPE, 88 for 16.4–18.4 min in AMTP, 150 for 18.4–20.4 min in BA, 164 for 25.3–27.3 min in eugenol) were recorded.

2.5. Assay of antimutagenicity

The detection of antimutagenicity was based on UV-induced mutation that is mainly caused by the bulky DNA product, thymine dimer. The assay was carried out according to the following: UVC (254 nm; 20 J/m^2)-irradiated cell suspensions (1.5×10^9 cells/mL) of *E. coli* B/r WP2 (*trpE65*, repair-proficient) were diluted by a factor of 10 and 1×10^6 times with PBS and plated to detect revertants and survivors, respectively. 50 μL of the sample solution was dissolved in dimethyl sulfoxide, and thoroughly mixed with the following: 0.2 mL of the diluted UV-irradiated cells plus 2 mL of 0.7% molten top agar, plus 0.5 mL of PBS. This cell-cocktail mixture was poured onto semi-enriched minimal agar medium (SEM). The numbers of revertants and survivors were counted as colony-forming units on the same organized SEM plates after incubation at 37 °C for 2 days. The antimutagenicity was evaluated by determining the relative mutagenic activity (RMA, percent of control): *i.e.* the mutagenic activity adjusted by sample toxicity, that was calculated using the formula $[(Mt/Mc)/(St/Sc)] \times 100$, where *Mt* is the number of revertant colonies in the presence of the test sample; *Mc* is the number of revertant colonies in the absence of the test sample; *St* is the number of surviving colonies in the presence of the test sample, and *Sc* is the number of surviving colonies in the absence of the test sample. To identify an active sample, we used a criterion "IC_{50}" determining the lowest dose needed to acquire a 50% RMA, which was calculated from a linear regression derived from at least 15 points taken over five doses. A fraction was determined active if it met this criterion.

2.6. Assay of differentiation-inducing effect in human colon cancer cells

The RCM-1 colon cancer cell line was diagnosed as a well-differentiated rectum adenocarcinoma derived from a 73-year-old female human [7], and was obtained from Dr. H. Kataoka of the University of Miyazaki (Miyazaki, Japan). The RCM-1 cell line is characterized as a partially-differentiated type that is suitable to purify "differentiation inducers", because the RCM-1 cells spontaneously differentiate as determined by the formation of ducts resembling villiform structures. Chemical-induced differentiation further enhances the number and size of ducts above the background levels of the spontaneously differentiated RCM-1 cells [6]. Thus, the real-time morphological observation of duct formation enables a simple assay for a rapid assessment of cell differentiation properties of potential chemopreventive compounds, thus being an ideal assay system to effectively assess the most active anticarcinogenic compounds in plant extracts. RCM-1 cells (1×10^5) were each plated into 96-multi-well plastic culture plate. When RCM-1 cells were cultivated with samples for 3 days, the duct number were measured from digital photographs at 50× using a Zeiss Axiovert 25 microscope equipped with a CCD camera. Duct formation activity was calculated as the fraction of the solvent control. To identify an active fraction, we used a criterion that determined the lowest dose needed to induce a 50% increase of the number of ducts over the levels found in the control. This was calculated from a linear regression derived from at least 12 points taken over four doses. A fraction was determined active if it met this criterion.

2.7. Assay of oxygen radical absorbing capacity (ORAC)

Antioxidative activity was measured using the oxygen radical absorbing capacity (ORAC) assay according to the method of Huang et al. [8] with slight modifications. The ORAC assay can detect the scavenging effect of the AAPH-generated peroxyl radicals induced by a test sample, which is monitored by the prevention of the loss of degradation of the fluorescein by peroxyl radicals. In brief, fluorescein and AAPH were dissolved with 75 mM potassium phosphate buffer (pH 7.4); and test chemicals were directly dissolved in an acetone/water/acetic acid mixture (70:29.5:0.5, v/v/v), and then diluted with 75 mM potassium phosphate buffer (pH 7.4). 200 μL of fluorescein (94.4 nM), 20 μL of serially diluted test chemicals, and

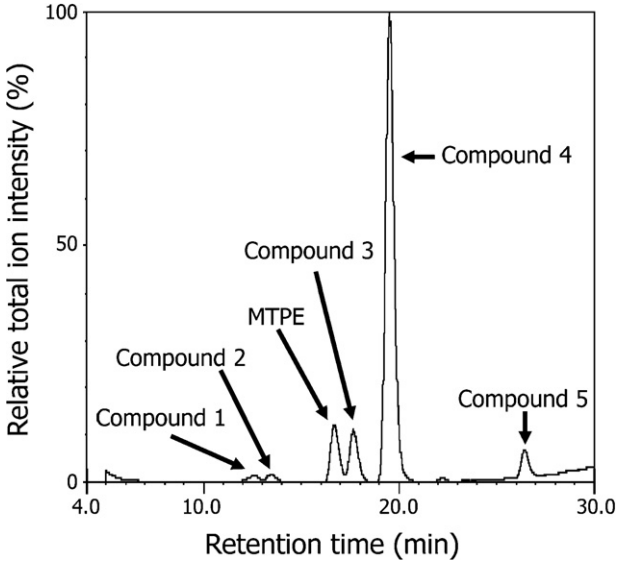

Fig. 1. GC–MS profile of fragrant ingredients in Katsura-uri. The capillary column in GC was a DB-5 (25 m × 0.2 mm, 0.33 μm film thickness). The column oven temperature was held at 60 °C for 5 min and then was increased to 250 °C at a rate of 5 °C/min.

75 μL of AAPH (31.7 mM) were dispended into 96-multi-well plastic plate. Trolox (50, 25, 12.5, 6.25 μM) solution prepared in 75 mM phosphate buffer (pH 7.4) was used to establish a standard curve. Degradation of the fluorescein was measured as a decline in fluorescence (Ex. 485 nm, Em. 520 nm) that was measured every 2 min for 90 min under 37 °C using a power scan HT microplate fluorescence reader (DS Pharma Biomedical, Osaka, Japan). The area under the curve (AUC) was calculated for each test chemical by integrating the relative fluorescence decline curve. The net AUC of the test chemical was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentration was determined, and ORAC values were expressed as μmol Trolox equivalents per 1 μmol of test chemicals using the Trolox standard curve.

3. Results

3.1. Identification of fragrant ingredients in Katsura-uri

A fragrant fraction (56 mg) was obtained by fractionating the *n*-hexane extract of fully-ripened Katsura-uri fruit on a silica-gel column and mobile phase containing 2.5% acetone in *n*-hexane. The fragrant fraction contained mainly six compounds as indicated on the total ion chromatogram obtained from a GC–EI-MS analysis (Fig. 1). One compound was MTPE (appearing at t_R 16.38 min), which was also previously identified by our group [6], while the remaining five compounds were new discoveries and were identified as follows. Compound 1 (MTAE), appearing at t_R 12.22 min showed the ion peak at an *m/z* 134 $(M)^+$, and prominent fragment ions with masses of 88, 61, and 45. Compound 2 (AMTE), appearing at t_R 13.20 min showed the ion peak at an *m/z* 134 $(M)^+$, and prominent fragment ions with masses of 91, 87, 74, 61, and 47. Compound 3 (AMTP), appearing at t_R 17.37 min showed the ion peak at an *m/z* 148 $(M)^+$, and prominent fragment ions with masses of 88, 61, and 47. Compound 4 (benzyl acetate), appearing at t_R 19.26 min showed the ion peak at an *m/z* 150 $(M)^+$, and prominent fragment ions with masses of 108, 91, 90, 79, 77, 65, and 51. Compound 5 (eugenol), appearing at t_R 26.22 min showed the ion peak at an *m/z* 164 $(M)^+$, and prominent fragment ions with masses of 149, 131, 121, 103, 91, 77, 65, and 55. To validate our tentative identification, we used authentic MTAE, AMTE, AMTP, benzyl acetate, and eugenol, which had the same retention times and *m/z* ratio of the ion peak as each of the tentatively identified compounds obtained from silica-gel chromatography. Thus, compounds 1–5 in the fragrant fraction of the *n*-hexane extraction of Katsura-uri were successfully identified

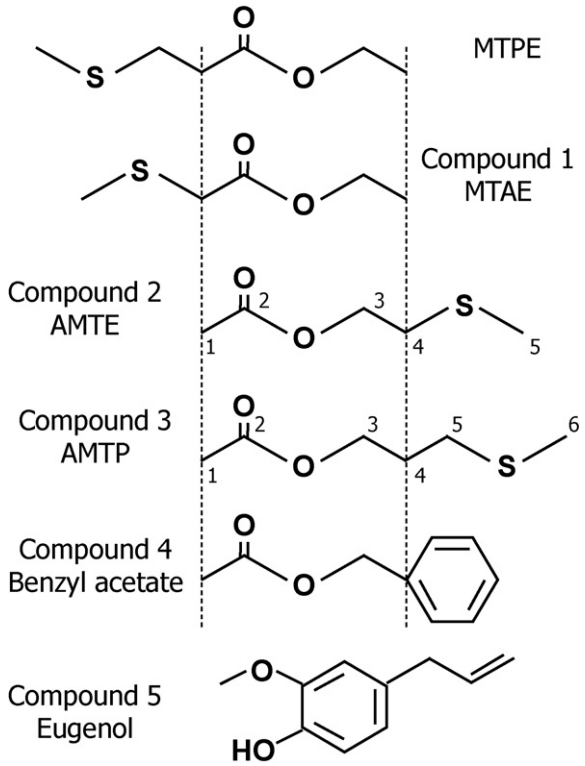

Fig. 2. Chemical structure of MTPE and compounds 1–5. MTPE: 3-methylthiopropionic acid ethyl ester, MTAE: methylthioacetic acid ethyl ester, AMTE: acetic acid 2-methylthio ethyl ester, AMTP: acetic acid 3-methylthio propyl ester. The inside broken lines were shown "$-CH_2-COO-CH_2-CH_2-$" structural motif.

to be those of MTAE, AMTP, AMTE, benzyl acetate, and eugenol. The chemical structure of MTPE and compounds 1–5 are shown in Fig. 2. Interestingly, MTPE and compounds 1–4 (MTAE, AMTE, AMTP, benzyl acetate) have a common structural motif consisting of "$-CH_2-COO-CH_2-CH_2-$". MTPE and compounds 1–3 (MTAE, AMTP, and AMTE) are all sulfur-containing compounds (Fig. 2), however, the sulfide on MTPE and compound 1 was on the acyl-group of the carboxylic acid side of the ester linkage, while the sulfide on compounds 2 and 3 was located on the alcohol side of the ester.

3.2. Fragrant character and concentration of MTPE and compounds 1–5 in Katsura-uri

MTPE and compounds 1–5 each had unique fragrances as determined by a human sense of smell (Table 1). MTPE, MTAE and AMTP caused fruity odors, and AMTE and benzyl acetate caused flowery odors. Eugenol is a principal odor ingredient of clove spice (*Eugenia caryophyllus*). Individually, MTPE, MTAE, and benzyl acetate were melon-like, apricot-like, and jasmine-like odors, respectively. In this paper, we used SIM mode of GC–EI-MS for more highly sensitive quantification analysis of MTPE and compounds 1–5, rather than UV (215 nm) absorbance mode of reverse-phase HPLC that was used in our previous study [6]. Thereby the quantification limit of MTPE was improved from 0.8 μg to 8 ng/100 g, and the detection limit was from 0.2 μg to 2 ng/100 g. By using GC–EI-MS SIM mode, the most abundant ingredient in the fragrant fraction of fully-ripened Katsura-uri was benzyl acetate (51.8 μg/100 g), followed by AMTP (9.4 μg/100 g), MTPE (2.8 μg/100 g), and eugenol (0.57 μg/100 g), respectively (Table 1). MTAE and AMTE were below the detection limits of 2 ng/100 g and 8 ng/100 g, respectively, and below the quantification limit of 8 ng/100 g and 49 ng/100 g, respectively. The difference between the values in the limits of MTAE and

Table 1
Fragrant character and concentration of MTPE and compounds 1–5 in Katsura-uri.

Chemical	Fragrant character	Katsura-uri (fully-ripened) (μg/100 g)	Katsura-uri (mid-ripened) (μg/100 g)
MTPE	Melon	2.8 (1.5–6.9)	0.057 (0.04–0.07)
Compound 1 (MTAE)	Apricot	[a]Lql	[b]Ldl
Compound 2 (AMTE)	Flowery	[a]Lql	[b]Ldl
Compound 3 (AMTP)	Fruity	9.4 (2.9–13.8)	[b]Ldl
Compound 4 (benzyl acetate)	Jasmine	51.8 (32.2–78.6)	0.26 (0.12–0.46)
Compound 5 (eugenol)	Clove	0.57 (0.39–0.77)	0.05 (0.048–0.054)

Each value represents mean of nine individual Katsura-uri (fully-ripened), and three individual Katsura-uri (mid-ripened) with their range in parentheses.
[a] Lql means less than quantification limit (8 ng/100 g for MTAE; 49 ng/100 g for AMTE).
[b] Ldl means less than detection limit (2 ng/100 g for MTAE; 8 ng/100 g for AMTE and AMTP).

Table 2
Antimutagenic, differentiation-inducing, and antioxidative effects of MTPE and compounds 1–5.

Chemical	Antimutagenic effect (IC_{50}, mg/plate)	Differentiation-inducing effect (ED_{50}, mM)	Antioxidative effect (μmol Trolox equivalent)
MTPE	Inactive	0.71 ± 0.15	0.61 ± 0.02
Compound 1 (MTAE)	6.53 ± 0.63	0.61 ± 0.22	0.12 ± 0.01
Compound 2 (AMTE)	Inactive	Inactive	0.44 ± 0.03
Compound 3 (AMTP)	4.44 ± 0.30	Inactive	0.67 ± 0.03
Compound 4 (benzyl acetate)	Inactive	Inactive	<0.001
Compound 5 (eugenol)	Inactive	Inactive	2.47 ± 0.07

IC_{50} values of antimutagenic effect and ED_{50} values of differentiation-inducing effect represents mean ± 95% confidence interval. Antioxidative effects are presented as ORAC (μmol Trolox antioxidative equivalent of 1 μmol test chemicals), and represents mean ± SEM.

AMTE was a function of the electron-ionization intensity. The levels of all six ingredients increased as a result of differences from mid-ripened to fully-ripened Katsura-uri. These results indicate that Katsura-uri begins to produce those fragrant ingredients between the mid-ripening to fully-ripening stage of fruit development.

3.3. Antimutagenic effect of MTPE and compounds 1–5

We determined the antimutagenic effect of MTPE and compounds 1–5 (using authentic chemicals) as a function of their ability to inhibit the UV-induced mutation in repair-proficient *E. coli* B/r WP2. Therefore, the assay detects the enhancing effect of suppression of mutation either by increasing the level of error-free DNA-repair for UV-induced DNA lesions (thymine dimers), or by increasing the opportunity for DNA repair by delaying DNA replication and mutation-fixation [9]. Compound 1 (MTAE) and 3 (AMTP) were antimutagenic, while the other four compounds showed no antimutagenic activity (Table 2). This is the first report on the antimutagenic activity of MTAE and AMTP. The antimutagenicity of MTAE and AMTP were dose-dependent, and the IC_{50} of MTAE and AMTP were 6.53 and 4.44 mg/plate, respectively (Fig. 3). Typical IC_{50} values for this assay range from 0.02 to 10 mg/plate [9], which indicated that MTAE and AMTP were on the higher end of this range, thus lacking very strong antimutagenic potential.

3.4. Differentiation-inducing effect of MTPE and compounds 1–5

Authentic chemicals of MTPE and compounds 1–5 were used in the induction of differentiation assay cell system. MTPE and compound 1 (MTAE) both induced differentiation of RCM-1 cells above the background of spontaneous differentiation (Fig. 4). The other four compounds did not induce the differentiation of RCM-1 cells above the background of spontaneous differentiation (Table 2). The differentiation-inducing effect of MTPE and MTAE were dose-dependent, and the ED_{50} of MTPE and MTAE were 0.71 and 0.61 mM, respectively (Fig. 4). Although MTPE, MTAE, AMTE and AMTP all contained a sulfide linkage and "$-CH_2-COO-CH_2-CH_2-$" motif, only MTPE and MTAE exhibited differentiating-inducing properties (Fig. 2). Apparently, the location of the sulfide linkage is important, considering that these two compounds had the sulfide located on the carboxylic acid side of the ester, while the inactive, AMTE and AMTP, had the sulfide located on the alcohol side of the ester. Benzyl acetate and eugenol, also did not induce differentiation above background, and like AMTE and AMTP, lacked a sulfide linkage on the carboxylic acid side of the ester. This differentiating effect of MTPE was previously reported by us [6], but this is the first report that MTAE can induce differentiation above background in the RCM-1 cancer cells.

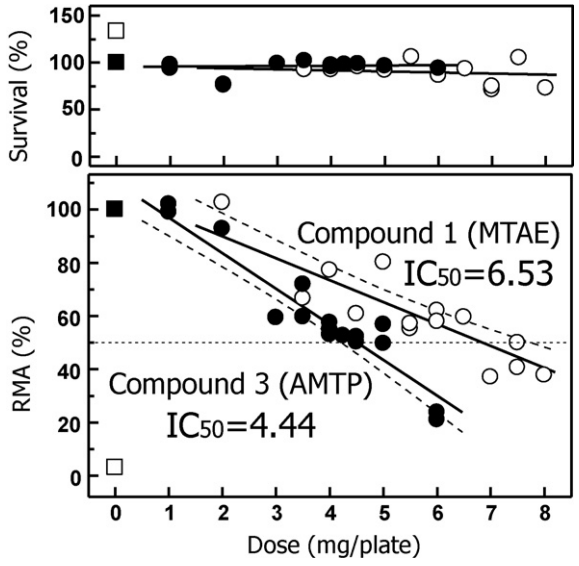

Fig. 3. Antimutagenicity of compound 1 (MTAE) and compound 3 (AMTP) on UV-induced mutation in *E. coli* B/r WP2. Cell suspensions of *E. coli* in a Petri dish were irradiated with a germicidal lamp at a dose of 20 J/m^2. The cell suspension (3×10^7 cells for mutant cell detection; 3×10^2 cells for viable cell detection) was poured onto SEM plate with soft agar containing MTAE or AMTP. The number of mutant cells and viable cells were determined by colony formation on SEM plates. The number of mutant colonies and survival colonies were 247 ± 6 and 212 ± 12 in UV-irradiated cells. Each point represents the individual value (15 of MTAE, 17 of AMTP) from three different experiments. A linear regression and its 95% confidence interval were expressed with solid and dotted lines. (□) Negative control (without UV-irradiation); (■): Positive control (with UV-irradiation); (○) MTAE; (●) AMTP

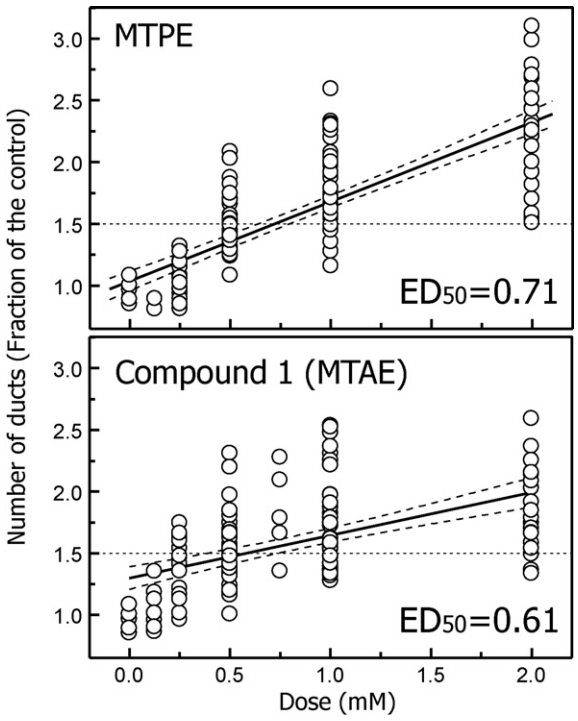

Fig. 4. Differentiation-inducing effect of MTPE and compound 1 (MTAE) in RCM-1 cells. RCM-1 cells (1×10^5) were seeded into 96-multi-well plastic culture plate. The cells were treated with MTPE and MTAE (0.125–2.0 mM) for 2 days from the next day of seeding. The formation of ducts was visually determined using phase contrast microscopy and the resulting images were used for quantifying the number of ducts in each culture plate. The data was plotted as a fraction of the control for number of ducts. Each point represents the individual value (162 of MTPE, 144 of MTAE) from four to six different experiments. A linear regression and its 95% confidence interval were expressed with solid and dotted lines.

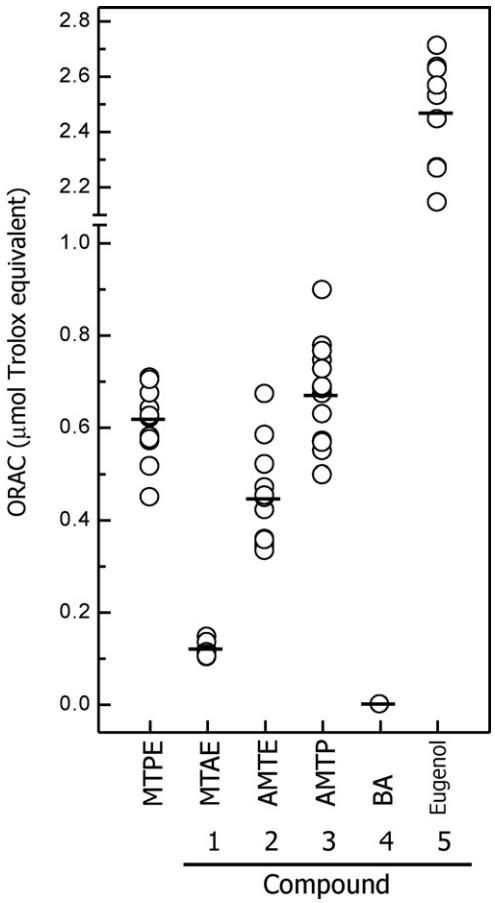

Fig. 5. Antioxidative effect of MTPE and compounds 1–5. Antioxidative effects are presented as ORAC (μmol Trolox antioxidative equivalent of 1 μmol test chemicals). Each point represents the individual value (13 of MTPE, 8 of MTAE, 15 of AMTP, 12 of AMTE, 6 of benzyl acetate, 9 of eugenol) from 3 to 12 different experiments. Each horizontal bar shows the mean.

3.5. Antioxidative effect of MTPE and compounds 1–5

The oxygen radical absorbing capacity (ORAC) was used to determine the antioxidative potential of MTPE and compounds 1–5 (using authentic chemicals). All compounds, except compound 4 (benzyl acetate), exhibited ORAC (Fig. 5). The most effective ingredient, compound 5 (eugenol), possessed 2.47-fold higher ORAC than Trolox, which was the positive control used in generating the standard curve for ORAC (Table 2). Eugenol is a principal compound in clove spice, and the extracts of clove are known to possess high ORAC [10]. Katsura-uri fruit also starts to produce eugenol when it reaches the fully-ripened stage (Table 1), and therefore the high ORAC can be obtained from fully-ripened Katsura-uri fruit. The antioxidative vitamin, ascorbic acid, had an ORAC of 0.32 ± 0.01 μmol Trolox equivalent (data not shown), which was significantly lower than three of the sulfur-containing esters [MTPE, compound 2 (AMTE), and compound 3 (AMTP)] (Table 2). Thus, these compounds can be considered to be strong antioxidants. Benzyl acetate, like MTPE, MTAE, AMTE and AMTP, possessed the "$-CH_2-COO-CH_2-CH_2-$" structural motif, but unlike these compounds, it did not have a sulfide, which indicates that the sulfide was probably involved in the antioxidative activity of this class of compounds. This antioxidative effect of sulfides should be further validated by determining ORAC with other compounds possessing sulfhydryl and alkylthio moieties.

4. Discussion

A GC–EI-MS method was successfully used to identify five new ingredients (MTAE, AMTE, AMTP, benzyl acetate, eugenol) in the fragrant fraction of fully ripened Katsura-uri fruit in addition to the previously identified, MTPE [6]. MTPE and the five new compounds were formed primarily in the ripening stage of the Katsura-uri fruit and contributed to the ripening fragrance of this fruit, each with their unique fragrant odor. All of these compounds exhibited one or more biological effects that could potentially contribute to the prevention of cancer. The biological effects determined were specifically antimutagenic, differentiation-inducing, and antioxidative activities. Because the highest levels of all these compounds were at the ripened stage, the greatest health benefits relevant to cancer would be at this stage of fruit development.

Of the six compounds, only MTAE and AMTP were antimutagenic. To calculate the contribution of antimutagenicity of MTAE and AMTP to the *n*-hexane extract of Katsura-uri is important to know whether the antimutagenicity of MTAE and AMTP represent entire antimutagenicity of the *n*-hexane extract; other antimutagens and synergistic compounds could be present in the extract. It can be calculated by a criterion, "yield/IC_{50}" value that allowed us to compare the comprehensive antimutagenicity with the quantitative aspect of the extract [5,9]. On the basis of this criterion, the yield (mg)/IC_{50} (mg/plate) for MTAE and AMTP were 0.012 (0.08/6.53) and 0.021 (0.094/4.44), respectively. However, the yield/IC_{50} of the *n*-hexane extract used in this experiment could not be calculated, because the lowest relative antimutagenic activity (RMA) was 52.6% at 10 mg/plate, and IC_{50} can not be obtained. In our previous study (1996–2009), the IC_{50} of the *n*-hexane extract of fully-ripened Katsura-uri ranged from 0.78 mg/plate to null value (>10 mg/plate, because the RMA did not reach 50% at 10 mg/plate). This variance

is probably dependent on biochemical changes occurring between the time that the sample was extracted until the time that the assay was performed. Some antimutagenic compounds are artifactually produced from thioesters in the *n*-hexane extract that did not show antimutagenicity (*e.g.* MTPE and AMTE) during the storage period. This is likely to be the reason of the wide-range of variance for the IC_{50} values of the *n*-hexane extract of fully-ripened Katsura-uri. In future studies, the comprehensive antimutagenicity of Katusra-uri should be confirmed by comparing the criterion yield/IC_{50} with MTAE, AMTP, and other artificially produced antimutagens.

A limited number of sulfur-containing compounds, such as isothiocyanates and thiosulfonates, specifically 4-methylthio-3-butenyl isothiocyanate and *S*-methyl methanethiosulfonate have been shown to exhibit antimutagenic activity in *E. coli* B/r WP2 [11,12]. These results along with our results indicate that sulfur containing compounds, including sulfides, have the potential to prevent the mutations. Considering that sulfur-containing esters are common in fragrant plants, future research in identifying new antimutagenic compounds could focus on fragrant plants. MTAE and AMTE have similar compositional formula to each other but were also structurally similar to MTPE and AMTP, yet these latter two compounds were not antimutagenic (Fig. 2). Partition coefficients (log *P*) were calculated for these compounds using Chemdrow 8.0 software (Hulinks Inc.). log *P* is a common parameter used to predict the hydrophilic/hydrophobic nature of a chemical substance (a high log *P* value indicates a hydrophobic chemical and vice versa). The predicted values for the antimutagenic compounds, MTAE and AMTE, were 0.68 and 0.52, respectively; and for the compounds lacking antimutagenic activity, MTPE and AMTP, the log *P* values were 0.97 and 0.63, respectively. Apparently there was no correlation of antimutagenic activity with log *P* values. Thus, neither chemical structure nor the hydrophilic/hydrophobic activity of these compounds predicted the antimutagenic properties of these compounds.

However, the ability of these compounds to induce differentiation of the RCM-1 cancer cells above the background of spontaneous differentiation could be predicted by chemical structure. Those that induced differentiation, MTPE and MTAE, are esters with a thiocarbonic acid and alkyl alcohol, while those that did not induce differentiation, AMTE and AMTP, are esters with a carbonic acid and thioalkyl alcohol. Our results indicate that esters with thiocarbonic acid and alkyl alcohol are effective in the induction of differentiation in RCM-1 cancer cells, and these types of esters should be further assessed for their underlying mechanisms.

A common structural motif was also predictive of the antioxidative activity for four of the compounds (MTPE, MTAE, AMTE, and AMTP). All of these antioxidative compounds possessed a sulfide linkage, while benzyl acetate, which did not exhibit antioxidative activity, lacks a sulfide group, although it did possess the "$-CH_2-COO-CH_2-CH_2-$" motif common to MTPE, MTAE, AMTE and AMTP. The sulfide linkages that were two carbons away from either the carbonyl (MTPE) or the oxygen of the ester group (AMTE and AMTP) had the highest antioxidative activity; while the sulfide group only one carbon away from the carbonyl group (MTAE) had the lowest antioxidative activity. However, eugenol had the highest antioxidative activity (2.47 μmol Trolox equivalent), and this compound was significantly different in chemical structure from the other five compounds. To rank the overall biological potency of the fruit, the following equation should be used, "ORAC activity × amount". Using this equation, the calculated values for AMTP and eugenol are 6.3 (0.67 × 9.4) and 1.4 (2.47 × 0.57), respectively. Thus, AMTP has 4.5-times higher antioxidative activity in the fully ripened fruit of Katsura-uri than eugenol. Similarly, MTPE also exhibited high antioxidative activity in the fully-ripened fruit of Katsura-uri, with an overall value of 1.7.

In summary, the use of a GC–MS method for fragrant ingredients analysis, allowed for the identification of six compounds (MTPE, MTAE, AMTE, AMTP, benzyl acetate, eugenol) that were uniquely up-regulated in the fully ripening stage in Katsura-uri pickling melon. Except for benzyl acetate, all the identified compounds possessed at least one or more anticarcinogenic effect as determined by antimutagenic, differentiation-inducing, and antioxidative activities. MTAE was the only compound exhibiting all three anticarcinogenic activities, while MTPE and AMTP possessed two out of three, and AMTE and eugenol possessed one out of three of these anticarcinogenic activities. The differentiation-inducing effect of MTPE was previously reported [6], but all other activities for this compound have not been previously reported, and all of these biological activities for the remaining compounds have yet to be reported. Our results indicate that the fully-ripened Katsura-uri fruit could potentially contribute to the prevention of human cancers.

Conflict of interest statement

The authors declare no conflict of interest.

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